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Meiosis Activating Sterols (MAS) and Fertility in Mammals and Man

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ABSTRACT In mammals two meiosis activating sterols (MAS) have been found to activate meiotic resumption in mouse oocytes, in vitro. FF-MAS (4,4-dimethyl-5α-cholesta-8,14,24-triene-3β-ol) was extracted from human preovulatory follicular fluid and T-MAS (4,4-dimethyl-5α-cholest-8,24-diene-3β-ol) from bull testicular tissue. Quite unexpected, these two sterols, which introduce the cholesterol biosynthetic pathway from lanosterol, may be locally acting substances with important physiological function for reproduction. FF-MAS and T-MAS are present in the preovulatory follicular fluid of different mammalian species and have the capacity to initiate resumption of meiosis in mouse oocyte cultured in the presence of hypoxanthine, a natural meiosis maturation inhibitor. FF-MAS is produced by the cumulus cells of intact oocyte-cumulus complexes upon FSH-stimulation and provides the oocyte with a go-signal for the resumption of meiosis. T-MAS constitutes the vast majority of MAS found in the mammalian testis and in the human ejaculate; in particular a high concentration is found in the spermatozoa. T-MAS may be produced by the spermatids and the presence of T-MAS in spermatozoa may suggest that T-MAS plays a role in fertilization by affecting the second meiotic division. *J. Exp. Zool. (Mol. Dev. Evol.) 285:237–242, 1999.* © 1999 Wiley-Liss, Inc.

Meiosis is a prerequisite for sexual reproduction in animals and is unique to germ cells to form haploid, genetically balanced gametes. In mammals all oogonia enter meiosis early in life and are transformed into oocytes when they enter meiosis. The oocytes are arrested in the late prophase of the first meiotic division, at the diplotene stage, almost simultaneously with their enclosure in follicles together with the granulosa cells. Meiosis does not resume until the oocyte-follicular unit has grown and reached a certain stage of maturity where it can respond to the preovulatory surge of gonadotropins, luteinizing hormone (LH) and follicle stimulating hormone (FSH), by resumption of meiosis and ovulation. By resumption of meiosis the nuclear membrane or germinal vesicle breaks down (GVBD).

The spermatogonia do not enter meiosis until puberty. In contrast to the female, only a fraction of the spermatogonia embark on meiosis leaving a mitotically dividing stem cell population for recruitment of new meiotic waves throughout adult life.

The mechanisms that initiate meiosis in the oogonia and the spermatogonia are unknown, whereas the processes involved in oocyte resumption of meiosis and in later stages of spermatogenesis have been clarified to a certain degree. Generally, gonadotropins are needed for the production of local intra- and extracellular signals that guide the resumption of oocyte meiosis in the female and the formation of spermatozoa in the male.

In mammals two meiosis activating sterols (MAS) have been found to activate meiotic resumption in mouse oocytes in vitro (Byskov et al., '95). FF-MAS (4,4-dimethyl-5α-cholesta-8,14,24-triene-3β-ol) was extracted from human preovulatory follicular fluid and T-MAS (4,4-dimethyl-5α-cholest-8,24-diene-3β-ol) from bull testicular tissue. Quite unexpected, these two sterols, which introduce the cholesterol biosynthetic pathway from lanosterol (Schroepfer, '82), may be locally acting substances with important physiological function for reproduction (Yding Andersen et al., '99).

The following sections give a summary of recent results of studies on MAS, the synthesis and localization in the gonads, the relation of MAS to

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the resumption of meiosis in oocytes and to spermatogenesis, and finally the possible role of MAS in fertility.

SYNTHESIS AND LOCALIZATION OF MAS IN THE GONADS

FF-MAS and T-MAS are synthesized by cytochrome P450 14α-demethylase (P450-14DM) which converts lanosterol to FF-MAS and sterol Δ14-reductase (Δ14R) which converts FF-MAS to T-MAS (Schroepfer, '82; Aoyoma et al., '86) (Fig. 1). FF-MAS and T-MAS are isolated from gonadal tissue by extraction and chromatographic isolation procedures (Baltsen and Byskov, '99). Adult mammalian testes mainly contain T-MAS, whereas T-MAS and MAS species are often found in equal concentrations in ovaries (Table 1).

MAS in the ovary

Both FF-MAS and T-MAS accumulate in preovulatory follicles of various mammalian species, e.g., in human (Baltsen and Byskov, '99) (Table 1). FF-MAS in human preovulatory follicular fluid is present in concentrations of around 1.3 μM and T-MAS in half of this concentration. In the puberal mouse ovary FF-MAS is absent or perhaps present in low levels but accumulates within a few hours in response to an hCG stimulation (Fig. 2). It is not certain which cells in the ovary are responsible for the gonadotropin induced MAS-accumulation. One way to study endogenous synthesis/accumulation of MAS is to apply compounds that interfere with the enzymes involved in the synthesis of MAS in the gonadal tissue. AY9944 is a well-known inhibitor of Δ7-reductase, the enzyme which forms cholesterol from its immediate precursor in the cholesterol biosynthesis (review Shefer et al., '98). It also inhibits Δ14R activity thus lowering conversion of FF-MAS to T-MAS and when added to microsomal fractions of liver cells, cholesterol decreases and FF-MAS accumulates (Kim et al., '95).

Recently, we found that AY9944 stimulates cultured mouse cumulus enclosed oocytes (CEO) to resume meiosis in a dose-dependent manner. In contrast, no such effect was observed in cultures of naked oocytes (NO) implying that the target enzyme(s) for AY9944 are localized in the cumulus cells (Leonardsen et al., '99). We showed that the cumulus-oocyte complex (COC) in fact synthesized lanosterol, FF-MAS, T-MAS, and cholesterol from the sterol precursor mevalonate, and that AY9944 caused accumulation of FF-MAS. The affect by AY9944 was abrogated when oocytes were split from their cumulus cells and joined for coculture. Thus, the physical interaction between oocyte and cumulus cells is pivotal for initiation of meiotic resumption.

These and previous studies using FSH stimulation of CEO (Downs et al., '88; Guoliang et al.,

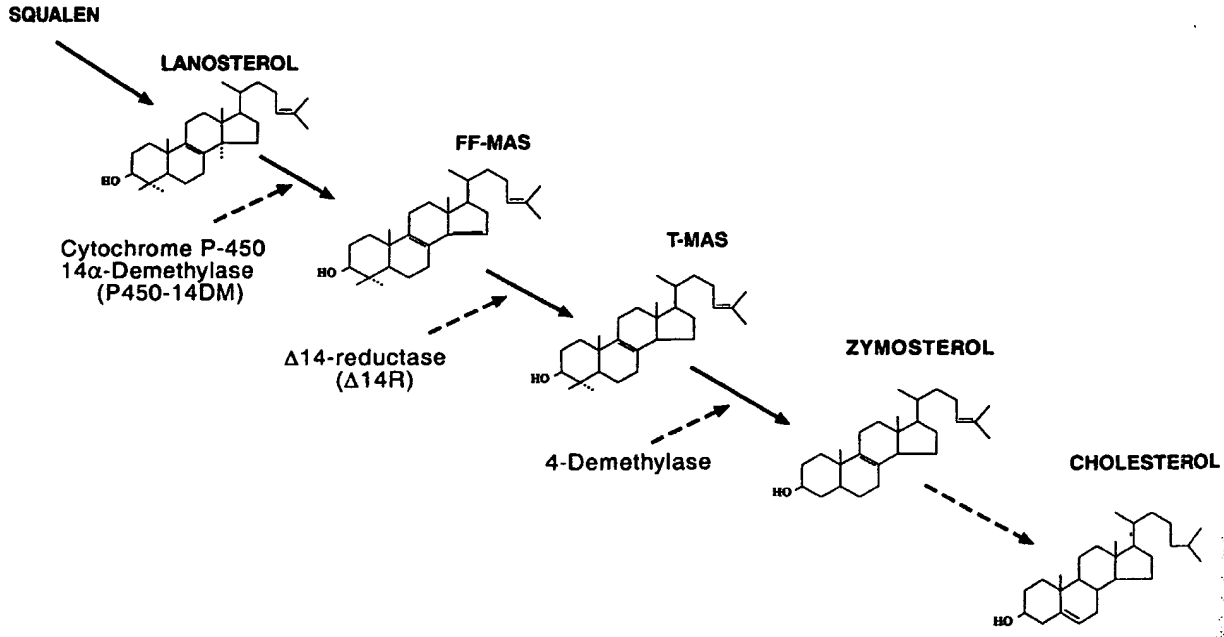

Fig. 1. FF-MAS and T-MAS are intermediates in the cholesterol biosynthesis. The cholesterol synthesis from squalen over FF-MAS, T-MAS, and zymosterol and the enzymes involved is shown. Modified from Aoyama and Yoshida ('86).

TABLE 1. MAS-concentration in different tissues and fluids

Source	Species	MAS (ppm)
Testicular tissue	Bull	>30
	Mouse	>30
	Stallion (normal)	>30
	Stallion (chryptorchid)	10–1
Testicular tubules	Rat	>15
Ejaculate	Human	<0.5
Spermatozoa	Human	>2
Ovary (CG-treated)	Mouse	>1
Ovary (not treated)	Mouse	<0.1
Follicular fluid	Human (preovulatory)	1
	Mare	0.1–0.3
	Pig	0.2

'94; Byskov et al., '97) support the idea that FF-MAS is produced by the cumulus cells, controlled by the oocyte itself, and that FF-MAS may play a central role in oocyte resumption of meiosis.

Recently another widely used enzyme inhibitor, ketokonazole, was reported to have no effect on oocyte maturation, in vivo in gonadotropin stimulated rats, or on spontaneously maturing oocytes, in vitro, whereas the compound affected the ovary by reducing the progesterone production (Tsafriri et al. '98). Ketokonazole is known to inhibit different cytochrome P450-enzymes, many of these involved in steroid synthesis, e.g., CYP11A1 (Gal et al., '94), and to interfere with certain hormone receptor proteins (Takahashi and Breitman, '92), and to inhibit

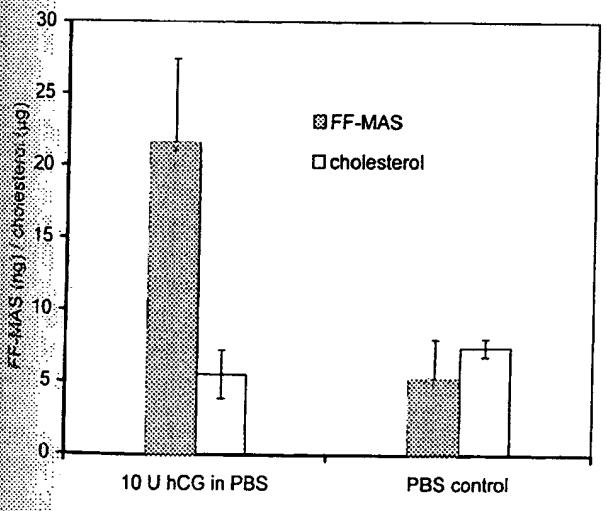

Fig. 2. Induction of MAS accumulation in mouse ovaries by hCG. Twelve immature female mice were stimulated to superovation. Forty-two hours later, 2 × 3 mice were given an ovulatory stimulus (10 U hCG in PBS, i.p.) and 2 × 3 mice were given vehicle alone (PBS, i.p.). The mice were killed 5 hr after hCG/PBS treatment and ovaries were prepared and analyzed for MAS and free cholesterol.

the enzyme P450-14DM which converts lanosterol to FF-MAS (see above). Another study showed that P450-14DM increases in rat ovaries after gonadotropin stimulation (Yoshida et al., '96) and MAS accumulates in gonadotropin stimulated mouse ovaries (Fig. 2). The stimulation of P450-14DM by gonadotropins thus opposes an inhibitory effect by ketokonazole on this enzyme in the living animal. The situation is different when oocytes resume meiosis spontaneously without exposure to gonadotropins. A resent study shows that spontaneous resumption of meiosis uses different signal transduction pathways than hormone-induced oocyte maturation (Leonardsen, '99. Obviously, further studies are needed to understand the physiological and cellular mechanisms involved in gonadotropin stimulated MAS-accumulation.

MAS in the testis

In contrast to the ovary, the vast majority of MAS species in the mammalian testis is T-MAS. Whereas FF-MAS is only present in trace amounts, T-MAS has been measured in concentrations of around 30 µg/g (ppm) testis-tissue of adult bull, horse, man, mouse, and rat, as well as in human spermatozoa (Table 1). In contrast, MAS concentration (ppm) is much lower in human ejaculate, in cryptorchid testes of the stallion in which spermatogenesis is abrogated, and in follicular fluids of different mammals. In a preliminary report it was proposed that MAS might be involved in initiation of meiosis as evaluated by meiotic initiation in male germ cells of cultured fetal mouse testes (Byskov and Yding Andersen, '95). However, the solubilization and transport of these very lipophilic substances make it difficult to draw conclusions on the possible role of MAS in initiation of meiosis and whether MAS is involved in meiotic initiation is still uncertain. In the puberal and adult rat testis it was recently reported, that post-meiotic germ cells express P450-14DM mRNA and exhibited an elevated level of P450-14DM activity (Strömstedt et al., '98). This indicates that MAS sterols are synthesized by the post-meiotic male germ cells. Taken together, it seems that MAS could play a role in spermatogenesis and may therefore be important for male gametogenesis (Strömstedt et al., '98).

INTRAFOLLICULAR SIGNALS INCLUDING MEIOSIS ACTIVATING STEROLS, MAS, INVOLVED IN OOCYTE RESUMPTION OF MEIOSIS

During most of its life the oocyte is maintained in meiotic arrest within the follicle. When released

from the follicle the fully grown oocyte resumes meiosis spontaneously. All cells within the whole follicular compartment are interconnected through a complex network of gap junctions securing transport of substances between the granulosa cells, cumulus cells, and oocyte. The granulosa cells produce different molecules which prevent or delay untimely resumption of meiosis, such as cAMP (Dekel, '88), purines (Eppig and Downs, '84; Eppig et al, '85; Downs, '97), kit ligand (Ismail et al., '96). Meiotic arrest may be maintained by such meiotic preventing substances transported from the somatic cells to the oocyte via the gap junctional complex. However, none of these substances can prevent the effect of gonadotropins on oocyte resumption of meiosis in the preovulatory follicle. Interestingly, some of these molecules are up-regulated by gonadotropins, e.g., cAMP (Dekel, '88), whereas others are down-regulated as kit ligand (Ismail et al., '96).

The actual trigger of the resumption of meiosis in different animals seems to be paracrine hormones, e.g., progesterone in *Xenopus* (Schorderet-Slatkine et al., '78). In mice MAS activates meiotic resumption in vitro (Byskov et al., '95; Grøndahl et al., '98; Ruan et al., '98). MAS may belong to the specific paracrine acting hormones that play important roles in meiotic resumption in other animal classes.

In mouse oocytes cultured with hypoxanthine, both FF-MAS and T-MAS overcome the inhibitory effect of hypoxanthine when added to the culture medium and induce resumption of meiosis in a dose-dependent way in concentrations between 0.03 and 3 μM (Byskov et al., '95). Cultured oocytes arrested with IBMX or dbcAMP are also dose-dependently induced to resume meiosis by FF-MAS in vitro using concentrations of MAS in the similar ranges (Grøndahl et al., '98). In a follicle culture model, FF-MAS in a high concentration (60 μM) increased resumption of meiosis to a similar degree as LH (Hegele-Hartung et al., '98). These authors found a similar effect of MAS in an ovarian perfusion model.

Previous studies have shown that a meiosis promoting substance is produced by mouse cumulus cells of intact COC by stimulation with FSH but not LH (Downs et al., '88). In such complexes, FSH initiates the production of a heat stable meiosis activating substance, suggested to be FF-MAS, but only when the contacts between cumulus cells and oocyte are kept intact (Byskov et al., '97).

In mouse ovaries both FF-MAS and T-MAS accumulate shortly after gonadotropin stimulation (Fig. 2). Since both FF-MAS and T-MAS can induce a resumption of meiosis it is possible that they exert additive effects or substitute one for another.

The signaling pathways for MAS during oocyte resumption of meiosis are not clarified. Presumably, the mechanism of hormone induced oocyte maturation is mediated through a receptor protein, although the presumptive MAS receptor has not yet been identified. A candidate FF-MAS receptor was suggested to be the orphan nuclear receptor LXRα, which is transactivated by several oxysterols, including FF-MAS (Janowski et al., '96). However, none of these oxysterols were active in inducing resumption of oocyte meiosis excluding LXRα as the receptor for MAS (Grøndahl et al., '98). In addition, inhibition of transcription does not inhibit MAS induced resumption of meiosis in the mouse, ex vivo, which makes it unlikely that a nuclear receptor is involved in the cellular response to MAS (Ottesen et al., '98).

MAS AND SPERMATOGENESIS

In cultured fragments of rat testicular tubules containing specific stages of spermatogenesis (Parvinen and Ruokonen, '82) T-MAS was measured using chromatographic isolation procedures. These quantitative measurements revealed that tubules containing the spermatogenic stages IX–XI contained more T-MAS (around 50 μg/g tissue) compared to other stages (II–VI, VII–VIII, XII–I) (15–35 μg/g tissue) (unpublished). In earlier studies we observed that culture media (spent media), in which the similar fragments of testicular tubules had been growing, contained a meiosis inducing activity which triggered meiosis in germ cells of fetal mouse testes after culture in the spent media (Parvinen et al., '82). This bioassay showed that the highest activity was extracted from stage VII–VIII. Whether the factor(s) that initiated meiosis in the fetal male germ cells might be T-MAS is, however, not known. Neither do we yet know if T-MAS is involved in triggering meiosis or in other stages of spermatogenesis in the adult testis.

Recently it was discovered that human semen contains T-MAS and the major source resulted from the spermatozoa. We have estimated that each spermatozoa possesses around 1×10^6 molecules of T-MAS (Baltsen et al., '98). Preliminary immunohistochemical results indicate that MAS is confined to the head of the spermatozoa in rat.

That MAS may be produced by the sperm cell

was inferred by the above mentioned study of the rat testis in which mRNA for P450-14DM was specifically expressed in round spermatides (Strömstedt et al., '98).

The function of T-MAS in spermatozoa is not clarified, but it may play a role in the process of fertilization and resumption of the second meiotic division.

POSSIBLE ROLE OF MAS IN FERTILITY AND CONTRACEPTION

Studies in different species have shown that FSH as well as epidermal growth factor, EGF, promote oocyte maturation, in vitro and implantation rate (Morishige et al., '93; Singh et al., '93; Merriman et al., '98). In a recent prospective and randomized study comprising 57 women undergoing IVF treatment we observed that addition of FSH and EGF to the culture medium during fertilization augmented the implantation rate of human pre-embryos (Yding Andersen et al., '99). The addition of FSH together with EGF during fertilization resulted in accumulation of FF-MAS in the culture media that was not seen in the control media without FSH and EGF. We suggest that the combined action of FSH and EGF enhances nuclear and cytoplasmatic maturation of the oocyte and fertilization, perhaps by the endogenous accumulation of MAS. This mechanism may mimic the natural situation of the fallopian tube where fertilization and pre-embryo development takes place in the presence of EGF, follicular fluid containing FF-MAS, and spermatozoa containing T-MAS. Studies on mice also indicate that when oocytes were fertilized after culture with FF-MAS the fertilization rate increased by a factor of 2–3 compared to cultures without FF-MAS (Hegele-Hartung et al., '98). In fact, the addition of the sterol zymosterol, which is an intermediate between T-MAS and cholesterol, improves the formation of two-cell-preembryos in an in vitro maturation and fertilization mouse model (Table 2).

Prevention of endogenous MAS accumulation may result in the failure of fertilization and proper pre-embryo development, an action that might be used as a contraceptive treatment. If a receptor for MAS is present on the oocyte, resumption of meiosis may be prevented by the use of MAS antagonists. If the mature oocyte is the only target cell for MAS, the application of MAS-antagonists could result in prevention of resumption of meiosis in the mature oocytes. A MAS-antagonist may thus serve as a novel contraceptive drug with no effects on the steroid synthesis.

TABLE 2. Effect of zymosterol on mouse preembryo development to the 2-cell stage from in vitro maturation and fertilization of naked oocytes (NO) and cumulus enclosed oocytes (CEO)[1]

Zymosterol (μg/ml)	Control	0.01	0.1	1.0
NO	38	60	52	51
CEO	75	91	70	76

[1]Results given as percent 2-cells per total number of in vitro matured oocytes.

CONCLUSIONS AND FUTURE STUDIES

Meiosis activating sterols, FF-MAS and T-MAS, are present in the preovulatory follicular fluid of different mammalian species. Both FF-MAS and T-MAS have the capacity to initiate resumption of meiosis in mouse oocyte cultured in the presence of hypoxanthine, a natural meiosis maturation inhibitor. FF-MAS is produced by the cumulus cells of intact oocyte-cumulus complexes upon FSH-stimulation and provides the oocyte with a go-signal for the resumption of meiosis.

T-MAS constitutes the vast majority of MAS found in the mammalian testis and in the human ejaculate, in particular a high concentration is found in the spermatozoa. T-MAS may be produced by the spermatids and the presence of T-MAS in spermatozoa may suggest that T-MAS plays a role in fertilization by affecting the second meiotic division.

Future studies on MAS will focus on:

- Molecular mechanisms involved in inter- and intra-cellular signaling, including a putative MAS-receptor
- Role of T-MAS in spermatogenesis, fertilization, and early embryo development
- Role of FF-MAS (and T-MAS) in oocyte resumption of meiosis
- Endocrine regulation of MAS synthesis and accumulation.

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